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Via email to: niceatm@niehs.nih.gov

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Re: Federal Register Vol. 70, No. 238, pp 74533-4, December 12, 2006: NTP Interagency Center for the Evaluation of Alternative Toxicological Methods; Announcement of an Independent Scientific Peer Review Meeting on the Use of *In Vitro* Pyrogenicity Testing Methods; Request for Comments

Dear Dr. Stokes:

These comments are submitted on behalf of the more than 10 million U.S. members of the Physicians Committee for Responsible Medicine, People for the Ethical Treatment of Animals, the Humane Society of the United States, the Alternatives Research & Development Foundation, the American Anti-Vivisection Society, and the Doris Day Animal League. We appreciate the opportunity to review ICCVAM's recommendations for five *in vitro* pyrogenicity tests (IVPTs) conducted using either human whole blood or human monocytic cell lines, and to provide comments regarding ICCVAM's "Draft Test Method Recommendations" (Recommendations) and "Draft Background Review Document" (BRD) on these methods. These comments incorporate by reference an earlier submission dated January 17, 2006.

At the outset, it should be stated that the parties to this submission have always endeavored to regard ICCVAM and its member agencies as federal partners who share our commitment to reducing, refining, and ultimately replacing the use of animals in regulatory toxicology. However, the abbreviated number of methods reviewed by ICCVAM and accepted by federal agencies in recent years raises concern over the genuine commitment to progress in the 3Rs by some federal agencies and/or their representatives on ICCVAM. The pyrogenicity BRD and Recommendations currently under discussion represent a glaring case in point.

ICCVAM's Recommendations accept the use of IVPTs only for the detection of lipopolysaccharide-mediated (LPS) pyrogenicity induced by gram-negative bacterial endotoxins "in materials currently tested in the RPT" (rabbit pyrogen test). Thus, for practical purposes, ICCVAM's Recommendations do not support the use or regulatory acceptance of these methods for the detection of gram-positive bacterial, fungal, or viral pyrogens. Moreover, ICCVAM specifically states that it does not regard the IVPTs as full replacements for the Limulus amebocyte lysate (LAL). Its Recommendations further state that in order to be considered as potential replacements for the RPT for the detection of non-LPS-mediated pyrogenicity, "additional studies that include a broader range of pyrogenic materials are recommended...such studies should include parallel RPT testing." More specifically, "when a positive non-endotoxin-mediated RPT result is encountered, this same sample should be subsequently tested *in vitro*."[1]

Despite the extensive discussion of the 3Rs throughout the BRD and Recommendations, it is not clear if or how ICCVAM's Recommendations could contribute to a meaningful reduction in animal use in

pyrogenicity testing if in fact we are not looking to replace the BET and continued comparisons to—and confirmatory testing in—the RPT are required for these methods.

We therefore strongly urge ICCVAM to significantly revise its Recommendations and BRD to more accurately reflect the potential use of these methods as full replacements for both the LAL and RPT. The available evidence shows that the IVPTs are fully valid for the detection of all pyrogens. We also strongly encourage ICCVAM to delete the recommendation regarding the conduct of de novo RPTs to further demonstrate *in vivo/in vitro* concordance.

General Comments

There are a number of disadvantages to current pyrogen-detection methods. These have been discussed previously, but necessitate a brief mention. The RPT exposes live rabbits to painful or distressing experiences; requires trans-species extrapolation; is less sensitive than the human fever threshold;[2] and is ill equipped to handle substances such as cellular products, radiopharmaceuticals, certain biologicals, and medical devices. The *LAL* also requires species extrapolation, can only detect LPS, and cannot be used for substances that interfere with the clotting process, biologicals ,or the direct assessment of medical devices.

Despite references to the 3Rs, the RPT is still used extensively, especially for complex biologically derived products and end-product release testing. Indeed, it is estimated that up to 400,000 rabbits per year are used,[3] and the LAL, despite catch-and-release procedures, results in an approximate 15% mortality rate.[4] It is therefore imperative, for both ethical and scientific reasons, that both of these tests are replaced by the alternatives presented here for endorsement.

In addition to the obvious ethical advantages of human whole and/or cellular blood pyrogenicity tests, the IVPTs have numerous scientific advantages. The first is the elimination of species extrapolation issues, since the proposed test methods are direct *in vitro* models of the human fever response. Additionally, because the pyrogenic response is a blood-mediated reaction, IVPTs are not limited by potential *in vivo/in vitro* extrapolation considerations, as some *in vitro* tests might be. The IVPTs are sensitive and can detect all potential pyrogens, not only LPS. They can be used to evaluate traditional pharmaceuticals as well as medical devices, species-specific cellular/biological therapies, cell culture media, air quality assessments, and human serum albumin, among other materials. The IVPTs could also be easily adapted into species-specific pyrogenicity tests for veterinary products.

The methods presented to the panel have undergone a full quantitative validation study. The validation studies were conducted in order to certify the IVPTs as appropriate for replacement of both the RPT and the LAL. The concordances and sensitivities for all five human blood-based methods are over 90%; specificities are above 80%; and all methods demonstrate low false-positive and -negative rates.[5] In comparison, historical data from 171 rabbits were used to calculate a theoretical sensitivity of 57.9% and a theoretical specificity of 88.3% for the RPT.[4]

Clearly, the IVPT methods, after 20 years of research and refinement, are a wholly superior way to detect pyrogens in medicinal products. However, the animal protection community has serious concerns related to the duplication of review efforts, as evidenced by the time ICCVAM has taken to arrive at this point with the IVPTs. As discussed in another recent set of public comments, ICCVAM continues to invest substantial time and resources in what are regarded by many as redundant and unnecessarily duplicative evaluations of 3Rs methods that have already undergone successful validation, independent peer review, and/or international acceptance in other jurisdictions. We therefore question the value of subjecting the IVPTs to multiple peer reviews—particularly when the animal-based RPT and LAL have not been subject to a level of scrutiny even closely resembling that of an ECVAM or ICCVAM validation study.

Specific Recommendations

Accept IVPTs as full replacements for the LAL

It is unclear why ICCVAM has chosen not to consider the IVPTs as appropriate for replacement of both the RPT and the LAL. With the validation of the IVPTs using an endotoxin standard, the LAL has become redundant. If there are specific cases of which we are not aware that require the LAL, exceptions can be made, but surely for ethical and scientific reasons the IVPTs should in general replace the LAL.

Certify the IVPTs valid for the detection of all pyrogens; conduct a "retrospective validation," if needed.

The mechanism of action behind the detection of LPS in the LAL, and hence the reason for its pyrogen specificity, is unique to arthropods. The mechanism of action, if not the magnitude of response, behind the detection of pyrogens in the RPT and the IVPTs is the same. Since the RPT is currently used to detect all pyrogens, there is no biologically sound rationale to conclude that the IVPTs cannot also detect all pyrogens—at a level at least equivalent to the RPT. ICCVAM documents drafted for review today state as much.

Indeed, BRDs submitted by ECVAM, draft BRDs posted by ICCVAM, and other materials list between 15 and 30 published studies discussing the detection of pyrogens, including non-LPS pyrogens, in human serum albumin, pharmaceuticals, and other materials. Some studies used clinically positive materials, and some used comparisons to the traditional *in vivo* or an *in vitro* version of the RPT.[6-8] One of these studies[6] compared the WB/IL-1 IVPT and the RPT using 96 batches of parenteral pharmaceuticals. Of all test substances, only one tested positive in all three (RPT, LAL, and WB/IL-1) test systems. The remaining 95 were negative in all test systems. ECVAM has also provided detailed testing results of materials with the IVPT methods that were determined to be positive for pyrogenic activity during clinical experience. Results were favorable in all assessments.[4]

It is at best perplexing to see peer review reports and testing recommendations stop short of giving the IVPT methods full validated certification, and only recommend the use of these methods for the detection of LPS-mediated pyrogenicity. While most pyrogenicity is indeed related to LPS, the ICCVAM draft recommended test method uses and future studies virtually guarantee that the RPT will not be replaced in the foreseeable future, as it will be needed to certify regulated end products completely "pyrogen free."

Given the results of Jahnke[6] above, it is further difficult to envision the concurrent *in vivo/in vitro* study recommended by ICCVAM. Hundreds of rabbits could be used in an unnecessary quest to get enough non-LPS-mediated pyrogenicity reactions in rabbits to subsequently confirm using the IVPT methods.

For ethical reasons, the ECVAM validation did not include such concurrent testing. Instead, the study chose LPS, a model pyrogen, to represent the pyrogen reaction and validate the *in vitro* test systems. There is no scientific reason to suspect that the IVPTs will not detect the full range of pyrogens. Published evidence supports this hypothesis,[7-10] as does supporting evidence submitted by ECVAM in early 2006.[4] If necessary, a coordinated assessment of such evidence—a retrospective validation of sorts—should more than allay any concerns about the applicability of the IVPTs to all varieties of pyrogen.

Articulate more clearly a path to full replacement

Investments in IVPTs by industry and the public sector are increasing. At least one American company, Charles River Laboratories, has for some time offered an IVPT assay for use in the detection of the range of pyrogens for research use. At least 200 laboratories worldwide have worked with or offer similar assays. Faith in the continued growth of these methods is clearly held by industry, academia, and government alike. With approval and continued use, we are confident that the IVPT methods will become

the "Gold Standard" for human pyrogen detection. The ICCVAM recommendations as currently written will limit the usefulness of these assays, and fail to achieve real reductions in animal use in a timely manner. We urge ICCVAM to revise its Recommendations as outlined above—and offer detailed guidance on how prospective end-users can adopt the IVPTs and put them into immediate practice.

Thank you for your attention to these comments.

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The Humane Society of the United States

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References

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Dear Dr Stokes,

re: Independent Scientific Peer Review Meeting on the Use of *In vitro* Pyrogenicity Testing Methods, Bethesda, MD, Feb 6th 2007 – request for comments.

In accordance with the invitation issued 12th Dec 2006, we would like to submit some comments for your consideration, specifically to the document 'Draft ICCVAM Test Method Recommendations: I*n Vitro Pyrogenicity Test Methods*', dated 01 Dec 2006 (file PWGrec12016.pdf).

We submit these comments as independent developers of an alternative proprietary *in vitro* pyrogen test, or IVPT. The test has been developed by us at the Faculty of Pharmaceutical Sciences at the University of Copenhagen [1]. Our test differs from the five ECVAM 'interleukin' tests under consideration here in that it is based on the measurement of reactive oxygen species produced from terminally-differentiated cells derived from the human HL-60 promyelocytic leukemia cell line. Whilst we believe that our test has all the advantages claimed by the various ECVAM test methods over the RPT, and more besides, our comments here will be restricted to the ICCVAM evaluation of the validation status of these ECVAM tests and the draft recommendations for such test methods.

Comments to PWGrec12016:

1.1 Draft recommended test method uses

"While the scientific basis of these (ECVAM) test methods suggests that they have the capability to detect pyrogenicity produced by a wider range of pyrogens (i.e. those mediated by non-endotoxin sources), there is insufficient data to support this broader application."

It is very clear from the current literature, and indeed from our own experience of many years working with similar assays (PBMC/IL-1 and MM6/IL-6 assays), that of the five ECVAM tests under evaluation, only the MonoMac6 test has a relevant and useful sensitivity towards non-endotoxin pyrogens. However, this property of the MonoMac6 test does not yet appear to have been validated.
Since the aim of your evaluation is to find an appropriate replacement for the RPT, and that one of the principal strengths of the RPT is that it offers the possibility of detecting pyrogens that would otherwise be missed by the BET, we offer the comment that perhaps it should be considered essential that a suitable IVPT replacement for the RPT must be validated in respect of its ability to detect relevant non-endotoxin pyrogens.

1.2 Draft recommended Future Studies

We wholeheartedly agree with the recommendation that "additional studies that include a broader range of pyrogenic materials..." be conducted if any of the five test methods under consideration are to be considered as potential replacements for the RPT.
We also strongly agree with footnote (3), that "an international standard [for non-endotoxin pyrogens]" is needed in order to demonstrate the utility of these (and other) test methods for the detection of non-endotoxin pyrogens. We suggest that suitable sources of non-endotoxin standards for this purpose might include yeast, fungi and gram-positive bacteria e.g. *Candida albicans* and *Staphylococcus aureus* either as whole organisms or isolated components hereof as for instance LTA from *S. aureus*. We suggest these two because both pathogens are of clinical relevance.

Appendix A, 1.4.4: Similarities and Differences in the Endpoints of IPT Methods and Currently recognized Pyrogenicity Test Methods

"...the *in vitro* release of pro-inflammatory cytokines, such as IL-1β and IL-6, is intended to predict the onset of [an inflammatory response]"
Although we do not argue against the relevance of these endpoints *per se*, we feel that we must make the comment that simple serum-level increases in either one or both of these interleukins are not sufficient in themselves to predict either an inflammatory reaction or a febrile response [2]. We should also like to point out that, although the focus here is on production of interleukins in the tests being evaluated, there are other endpoints that are just as relevant for prediction of inflammatory responses by the human immune system, indeed perhaps more so, and that one of these is the production of reactive oxygen species by macrophage- and PMN-like cells when challenged with pyrogenic materials.

Appendix A, 2.3.1: Essential Test method Components, *In Vitro* Cell Culture Conditions

Regarding the use of cryo-preserved whole blood, we appreciate that this is one possible way to avoid the need to make large numbers of willing blood donors available to testing laboratories. However, several laboratories, including our own, have experienced significant problems using cryo-preserved blood in these assays – in our case, the "cryo WB/IL-1" test, commercially obtained from Charles River Labs. Whilst the WB/IL-1 test delivered the results expected using fresh whole human blood, when we tested the same kit with cryo-preserved blood obtained from a source recommended by the manufacturers, it gave no results at all. We believe that the reason for this was that the cryo-preserved blood cells had been irretrievably damaged by the freezing process; the blood sample, thawed according to instructions, was thick and denatured with every indication of extreme cellular damage. From our discussions with others who have also tried using cryo-preserved blood in this test, we conclude that this is a not un-common problem.

Appendix A, 2.3.3.2: Positive Control Substance:

An important distinction between the BET/LAL test and the RPT is that the BET detects only endotoxin pyrogens, whereas the rabbit pyrogen test is capable of also detecting non-endotoxin pyrogens. We suggest that it should therefore be a requirement of the performance standards for any IVPT that might replace the RPT that said *in vitro* test is assessed directly for its ability to detect non-endotoxin pyrogens, as well as LPS.
We therefore suggest that the performance standards include a requirement for one or more positive control pyrogenic substances selected from a group of non-endotoxin pyrogens (perhaps those suggested in our comment to point 1.2, above), in addition to the reference standard LPS to demonstrate adequate sensitivity of the cell system to relevant pyrogens. The sensitivity of any suitable test method to these non-endotoxin pyrogens should be at least comparable to the sensitivity of the rabbit pyrogen test to these same substances.

Appendix A, 2.4: Reference Substances for In Vitro Pyrogenicity Test Methods

In line with the various comments made above, we would suggest that Reference Substances be spiked not only with Gram-negative endotoxin standards, but also non-endotoxin pyrogen standards in order to properly assess the accuracy and reliability of a proposed IVPT that should replace the RPT.

We hope that these few comments will be useful to you in the process of evaluating the validation status of the EVCAM tests, and for drafting future Performance Standards by which to determine the relevance and reliability of these and other *in vitro* test methods for the highly desirable purpose of replacing the RPT.

Yours sincerely,

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P.S. In case this may be of interest, we have attached the most recent results obtained with our HL-60 ROS IVPT, further optimized from the test reported in [1]. The table reports the responses obtained from a wide variety of pyrogenic components. This table also contains results obtained by us for these same substances tested using the WB/IL-1 IPT (Charles River Labs), and literature data for the same substances run in the RPT.

References:

[1] Timm, M., Hansen, E.W., Moesby, L., Christensen, J.D. (2006). Utilization of the human cell line HL-60 for chemiluminescence based detection of microorganisms and related substances. *Eur J Pharmaceutical Sciences* **27**: 252-258

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Pyrogen Test Benchmark Data: Hansen & Timm, University of Copenhagen

Positive detections by four assays evaluated for pyrogen determination

Sample	HL-60 assay	IPT assay	Rabbit pyrogen test	LAL test
Zymosan 0,5 µg/ml	+	-	-	-
Zymosan 5 µg/ml	+	+	-	-
LTA standard (0,5 EEU/ml)	+	+		-
Candida albicans 10^4 yeasts/ml	+	-	-	-
Candida albicans 10^5 yeasts/ml	+	-	-	-
Saccharomyces cerevisiae 10^4 yeasts/ml	+	-	-	-
Saccharomyces cerevisiae 10^5 yeasts/ml	+	-	-	-
LTA from *Bacillus subtilis* 25 ng/ml	+	(-)	-*	-
LTA from *Bacillus subtilis* 100 ng/ml	+	+	-*	-
Staphylococcus aureus 10^5 bacteria/ml	+	-	-*	-
Staphylococcus aureus 10^6 bacteria/ml	+	-	+*	-
Bacillus subtilis 10^4 bacteria/ml	+	+	+*	-
Bacillus subtilis 10^5 bacteria/ml	+	+	+*	-
Salmonella typhimurium 10^3 bacteria/ml	+	(-)	-*	+
Salmonella typhimurium 10^4 bacteria/ml	+	+	+*	+
Aspergillus niger spores 10^5 spores/ml	+	-		-
Aspergillus niger spores 10^6 spores/ml	+	-		-
LPS standard 5 EU/ml	+	+	+*	+
LPS standard 2,5 EU/ml	+	+	+*	+
LPS standard 1 EU/ml	+	+	+*	+
LPS standard 0,5 EU/ml	+	+	+*	+
LPS standard 0,25 EU/ml	+	-	-*	+
LPS standard 0,125 EU/ml	+	-	-*	+

(-) samples do excite a response above non stimulated control, but do not score as pyrogenic according to manufactures description.

(*) data obtained from literature.

Rec'd 1/29/07

26 January 2007

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The following comments are made in response to: FR Notice (Vol. 71, No. 238, pp. 74533-74534, 12/12/06), Scientific Peer Review Meeting on the Use of In Vitro Pyrogenicity Testing Methods; Request for Comments.

We would like to acknowledge the efforts that NICEATM and ICCVAM have made towards implementing *in vitro* testing as a replacement for that of the standard *in vivo* methods for pyrogenicity. Towards this common goal we are all in agreement. However, even though we share the goal of replacement of methods, which use animals wherever possible, we in the medical device industry have had to continue to use the rabbit pyrogen test to assure that new material components for our products do not contain substances known as "material-mediated" pyrogens. The known substances of this type, listed in ISP 10993-11 Annex F, are generally chemicals, which are mostly understood to directly stimulate the thermoregulatory center in the brain to produce a pyrogenic response. This type of non-endotoxin pyrogen is rare, I have been working in the medical device industry now since 2004 and in this capacity have never observed a pyrogen test conducted on a medical device that did induce a febrile reaction in an animal. This testing is performed by government mandate: Code of Federal Regulations, Title 21, (21CFR610.13) and as such, is not an option for the medical device industry. Further, ISO 10993-11:2006 Annex F states that medical devices containing new chemical entities or substances which have previously elicited a pyrogenic response, should be evaluated for material-mediated pyrogenicity.

The proposed *in vitro* methods for assessing pyrogenicity do not include any data that would support the validity of these methods for the indication of material-mediated pyrogenicity. *In vitro* pyrogen tests appear from studies cited and summarized to be a suitable substitute for the LAL test for endotoxin testing (which we use routinely for product lot release) with additional capability to detect pyrogenic substances from gram positive cell walls and fungi; but it is mechanistically unlikely these methods can detect the majority of materal-mediated pyrogens (Annex F list), because there is no macrophage/ cytokine involvement. To accept any/all of these methods as replacements

for the rabbit pyrogen test in all cases without data to support their intended use/s for the acceptance of medical devices would at the very least be deemed to be an equivocal representation for safety considerations in human practices. A minimum consideration should be given to a further study to evaluate some of the non-endotoxin material-mediated pyrogens contained in Annex F of the ISO 10993-11 document by the *in-vitro* pyrogen methods. We strongly recommend that such a study be initiated.

The ICCVAM background document itself notes the following items of concern regarding the assays:

- One identified limitation of the *in vitro* methods is the lack of data to determine their responses to, and suitability for, non-endotoxin pyrogens that are known to be detected by the RPT.
- ECVAM validation studies focused specifically on Gram-negative endotoxin due to the unavailability of standardized, non-endotoxin pyrogens
- ***In vitro* pyrogenicity test method validation studies should evaluate an adequate sample of substances and products of the types that are intended to be tested with these methods. The list of test substances selected for inclusion in the ECVAM validation studies consists solely of marketed parenteral pharmaceuticals that have been labeled as free from detectable pyrogens. No specific rationale was provided for the selection of these test substances.**
- A recognized limitation of the *in vitro* methods is the lack of data to determine their responses to, and suitability for, non-endotoxin pyrogens that are known to be detected by the RPT.

Further testing should be conducted using a representative sample of the types of material-mediated pyrogens as are found in Annex F of ISO 10993-11:2006. When testing of this nature is completed, then the data generated would be better suited for justification of the assays acceptance in the medical device industry. Until such testing is completed and data becomes available, it would be extremely difficult to justify the use of these assays for medical devices.

Respectfully submitted by:

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